**Lab: Bacterial protein expression**

**Objective**

The purpose of this lab is to overexpress a recombinant protein in bacteria. In the course of this lab you will: transform competent bacterial cells with a DNA plasmid, grow a starter culture of bacteria; use this overnight culture to inoculate a larger culture and express the recombinant protein; harvest the cells and freeze them for additional experimental work in the next lab.

Note: your lab report will be cumulative over the next 3 wet labs (Expression, purification, characterization).

However, we will upload the images that you take in this lab to the Wikispaces website to share with the rest of the class. This will consist of the images with figure captions.

Note: Work in pairs for the first part, then as individuals

Note: Fill out your labnotebook as you go.

**Lab Safety**

You will be handling a lab strain of bacteria that is not harmful to adults. However the bacteria contain recombinant DNA and a gene for antibiotic resistance and must be handled properly. Wear glasses and gloves (optionally a lab coat) while handling the material and wash your hands after removing your gloves and before exiting the lab. The bacterial waste must be treated and disposed of properly. For liquids this entails dumping them in the labeled waste container that contains bleach. DO NOT DUMP MEDIA OR CULTURE DIRECTLY DOWN THE DRAIN WITHOUT TREATING WITH BLEACH for 20 minutes. Wash all glassware used by first rinsing with bleach then water and subsequently scrubbing clean with Alconox. Solid waste should be collected in the orange or red waste bags for autoclaving or collection by EHS, NOT in the regular trash.

**Background**

Lab strains of *Escherichia coli* bacteria have been developed that are used to produce proteins for experimental work. By overexpressing the protein of interest in bacteria, proteins that are in low abundance in their native organism can easily be purified for crystallography, enzyme inhibition assays, etc. This technique also allows one to study human proteins without having to use human tissues. Because molecular biology techniques (such as PCR or site-directed mutagenesis) allow the DNA to be manipulated, non-native proteins may also be produced. For example, you may want to test the effect of an amino acid mutation in an antibacterial resistant strain of bacteria.

We have obtained a plasmid (pGEM-gbr22) that encodes for a fluorescent protein originally cloned from a coral from the Great Barrier Reef. It is easy to follow the expression and purification of this protein since it is purple in color (most proteins are colorless in solution and can only be detected by following the absorbance at 280 nm in a UV spectrophotometer). The expression plasmid (shown to the right), which has been inserted into the *E.coli* BL21 host bacteria, carries a gene for ampicillin resistance and the fluorescent protein has six histidine residues appended at the C-terminus. The hexa-histidine tag is an affinity tag that allows for fast and efficient purification of the protein.

In this week’s lab you will transform competent cells, grow the *E.coli* cultures to express the purple protein, harvest the cells by centrifugation, resuspend them in buffer, and store them at -20 C. In the next 2 labs you will lyse the cells, purify the protein, and characterize the protein using gel electrophoresis and by UV-Vis spectroscopy.

**Aseptic technique**

When working with bacteria and bacterial media (or any type of cell culture), it is very important that your cultures and media do not become contaminated by other organisms. Because bacteria are ubiquitous and are found on fingertips, bench tops, etc., it is important to minimize contact with these contaminating surfaces. LB and SOC are nutritionally rich media and most bacteria and other microorganisms will thrive in them. Good aseptic technique is important in insuring consistent results when using protein expression techniques.

When working with cultures in test tubes or flasks, work as rapidly as is consistent with careful technique. Keep the tubes/flasks open a minimum amount of time. Label all cultures appropriately. Talking and movement should be minimized around open cultures and agar plates to avoid stirring up air currents that can deposit contaminants.

In general, the only lab items that are sterile are ones that are removed from sterile packaging (e.g. disposable pipettes, the packaging should state that it is sterile) or ones that have been sterilized by autoclaving or filter sterilization. The following items are NOT sterile: nanopure water, gloves, beakers, graduated cylinders, aluminum foil. Glassware and aluminum foil is sterile if it has autoclave tape that indicates it was autoclaved (e.g. visible black stripes).

**Prelab calculations**

1. Once the mentor tells you the stock concentration of plasmid DNA, calculate how many ul (microliters) you will need to have to get ~ 300 ng (nanograms) in your transformation.
2. Also, calculate how many ng (nanograms) actually get plated
3. Calculate the amount of your 50 mg/ml ampicillin stock solution to add to 5 ml of LB so that the final ampicillin concentration is 100 μg/ml.
4. Calculate the amount of your ampicillin stock solution to add to 25 ml of LB to make the final concentration 100 μg/ml.
5. Calculate how much of your 10x PBS stock solution is required to make 10 ml of a 1x working solution.

**Experimental procedure**

This lab will take several days to complete. Since our incubator space is limited, we will stagger groups to start on Monday, Tuesday and Wednesday.

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| **Day** | **Time** | **Procedure** | **Duration of prep time** |
| Day 1 | late afternoon or evening | overnight transformation | 2 hrs |
| Day 2 | morning | starter culture  8 hrs long | 1hr |
| Day 2 | evening | large culture  24-48 hrs long | 30 min - 1hr |
| Day 3 or  Day 4 | During Late afternoon  (if bacteria purple) | Harvest bacteria | 1.5 hr |

The first steps to over expressing a protein in bacteria are cloning the gene and inserting the gene into an expression plasmid. Then the presence of the gene is confirmed and the gene is usually sequenced to ensure the cloning process hasn’t introduced any mutations. These steps have already been done for you. The next step is transforming the expression strain of bacteria (in this case *E.coli* BL21(DE3)) with the plasmid. The bacteria are then grown on an LB agar plate with antibiotic to select for colonies that have the gene for antibiotic resistance.

Transformation of bacteria with a new plasmid is not 100% efficient - some bacteria will successfully take up the plasmid and others will not. Consequently, we need a selective marker to help us identify the bacterial colonies that have been successfully transformed so that we don’t grow up ‘empty’ bacteria which could compete with the transformed bacteria and overtake the colony – leaving us with none of our protein. There are many different selective markers and antibiotic resistance is our choice of selective markers. The gene conferring ampicillin resistance is cloned into the plasmid along with our gene of interest. When bacteria are grown on an LB agar plate or in LB broth containing ampicillin, only those bacteria that have been transformed with the plasmid containing the ampicillin resistance gene (and our gene of interest) will survive.

Competent cells are specially treated bacterial cells that are more capable of uptaking exogenous DNA. Usually this is achieved by preparing the cells with CaCl2 which helps coat the negatively charged DNA as it enters the cell through pores in the cell membrane. For this lab, we will be using BL21(DE3) competent bacterial cells from Sigma or NEB or Novagen. These are stored in the -80 oC freezer

**Transforming the competent bacterial cells (~ 2hrs)**

Work in **Pairs for the Transformation**

Materials: Ice bucket, 42oC water bath, gas burner, 2 x 14 ml clear, sterile round-bottom transformation tubes, 37oC incubator, colirollers, 2 LB Agar Amp plates, 1 spare Agar plate w/o antibiotic, competent cells on ice, plasmid DNA on ice, LB media, SOC media, pipette and pipette tips

Turn on water bath first. Then get ice.

There will be 3 plates: one experimental plate with DNA and one Control plate that has no DNA to confirm that we have a clean technique and no colonies are growing without the plasmid. Lastly, we will have one plate for ‘fun’ – this one you will sneeze on or take a swab from a disgusting place (e.g. the lab floor)! Since it will not have any antibiotic mixed in the agar – microorganisms such as yeast, bacteria and mold will be able to grow.

**Label plates** with the type of antibiotic (e.g. Amp or None - for the Fun plate), name of the organism e.g. BL21(DE3), plasmid vector name (e.g. pGEM-gbr22 or No DNA control), the date, VDS, and your 3-letter initials and your partner’s 3-letter initials. Be sure to write ‘Fun Plate’ on the fun plate.

The gas burner can be turned on to sterilize the general area when handing the bacteria – but it should be turned off during incubation periods. Burners should never be on when you leave the bench area!

Mentor will place a tube with 50 ul of competent bacterial cells in your ice bucket

(which brand did you get?)

These must be used within **5 minutes** after the tube thaws!

Add 25ul of bacteria (**HALF** of what you got) to each of the 2 transformation tubes

(one for DNA and one for Control)

Briefly spin down the tube of plasmid DNA using a mini-centrifuge on the benchtop

Add 1-2 ul of plasmid gently to bacteria in the **DNA tube only**. Control tube gets no DNA

This should be roughly ~300 ng of plasmid DNA

Wait 30 min on ice

While you are waiting, place your 3 plates in the incubator to pre-warm them

Heat shock tubes in 42 oC water bath for 45 seconds.

The duration of this heat shock is critical.

Wait 2 min on ice

Add 200 ul of **SOC** media (this is expensive and prone to contamination – treat well)

Shake in incubator for 30 min at 37 oC at about ~250 rpm (use dry shaker or water bath)

Sterilely place 5-6 colirollers onto each plate (DNA and Control)

Pipette 50ul of bacteria/SOC mixture from tube onto each plate

(one person spreads control, one person spreads the plasmid plate)

Roll plate around until bacteria is evenly spread (about 2 minutes)

Pour colirollers off to be saved and resterilized (in ‘USED COLIROLLERS’ bottle)

**Fun Plate**: Take the spare 3rd plate that has no antibiotic – cough or sneeze on it or take a swab from a disgusting place on it.

Cover plates with lid

Turn off the gas burner

Invert plates and store in 37oC incubator overnight

**Day 2 (morning): Growing a starter culture (30 min – 1 hr)**

Do this as **Individuals**

Materials: gas burner, 2 x 14 ml clear, sterile round-bottom transformation tubes, 37oC incubator, LB media, Ampicillin stock (50mg/ml)

The gas burner can be turned on to sterilize the general area when handing the bacteria.

For **Figure 1.** Take a picture of your plates using the digital camera (or cell phone). Your images need to be good enough so that you can see your colonies well.

Post the image online to your Protein Labs page on our Wikispaces site:

<http://vdsstream.wikispaces.com/Protein+Labs+SP13>

NOTE: make sure your plates have been properly labeled with a permanent marker so that it is obvious what they are in the image.

The starter culture will be grown in LB supplemented with 100 μg/ml ampicillin. Set up the burner to sterilize the general area and using good aseptic technique, add the appropriate amount of ampicillin to **TWO** tubes of 5 ml of LB in a sterile culture tube with cap. NOTE: The second one is a back-up. Be careful not to touch the interior of the tube with the body of the pipette (while pipette tips are sterile, the pipette itself is not). However, it is acceptable to touch the pipette tip to the inside of the tube to dispense the ampicillin. Then use a sterile pipette tip to pick a single colony of bacteria growing on the LB/agar plate and transfer the cells to your LB/amp media (– each person will pick one colony to make a total of two duplicate tubes). To pick colonies, you just gently swipe the surface of the agar plate without digging into the agar. The bacteria grow on the surface and not inside the agar. Then dip the tip into the tube of LB. The sterile pipette tip can be dropped in and left until you clean the container the next day. A bacterial culture will grow from a surprisingly small sample taken from an agar plate.

Place the cap loosely on the tube. Label the side of the tube with VDS, your initials, the organism: BL21(DE3) and plasmid: pGEM-gbr22, and the date. Make sure the burner is off. Place the tube into a rack in the shaking incubator and grow during the day (usually 8 hrs) at 37 C and 200-350 rpm. Make sure the incubator is turned ON and lid is closed properly.

Your plates can now be saved in the 4OC fridge. Wrap them in parafilm and store in VDS section. – you may leave the Fun Plate in the incubator to grow up more!

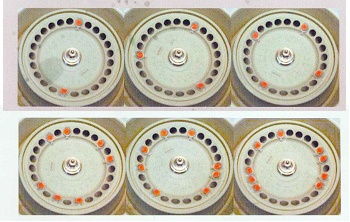
**Day 2 (Evening): Protein expression in large culture (~1 hr)**

Do this as **Individuals**

You will make the large culture for overnight expression. The bacteria should be in log phase growth from the starter culture. Being careful to avoid contamination, transfer 25 ml of fresh LB to a clean/sterile 125 ml Erlenmeyer flasks. Add ampicillin to the LB to make the final concentration 100 μg/ml. Use a piece of tape to label the flask with Date, VDS, your initials, the type of media (LB + Amp), the organism: BL21(DE3) and plasmid: pGEM-gbr22.

Transfer 0.625 ml of your starter culture from the tube to the 125 ml flasks, cover the flask with foil, and secure the flask into a clip in the shaking incubator (you may make two of these if we have enough flasks and shaker space).

* Verify that your flask is not loose when shaking. If so – fix it now.
* Ensure that the shaking incubator is operating at 37oC and 200-350 rpm and allow the culture to grow for 16-24 hrs. (If dry shaking incubator is broken, then use the water bath shakers).



**Day 3 or 4: Harvesting cells (Do this as Individuals)**

You will know when it has grown enough because the media should be turbid and **purple** in color as shown below in the picture of flask. If the culture is still yellow, leave in the shaking incubator and check after an additional 16-24 hrs. When the culture is purple, you are ready to harvest the cells.

For **Figure 2.** Take a picture of your purple culture in the flask and post to your Protein Labs page. Make sure your flask label is visible in the image.

The larger benchtop centrifuge (Allegra X-15, at the far end of the lab) will be used to harvest the cells. Turn on the centrifuge and set the temperature to 4oC.

Before spinning your cells down, take a 500 ul sample of your culture, dispense into a labeled Eppendorf tube, Label as **sample 1** and your initials and date and store at 4oC in the VDS tube rack. You will need this sample for the next lab (Characterization Lab where you run a gel)

EACH student should save their own samples for the gel so that you have your own in case your partner loses them or you pair up with someone else for the gel.

Pour your bacteria into a 50ml conical tube that has been labeled with your VDS, your initials, the type of media (LB + Amp), the organism: BL21(DE3) plasmid: pGEM-gbr22, and the date. Use another tube with an equal volume of water as a balance – there may be balance tubes by the centrifuge already. Use an electronic balance to determine when the masses are equal. Alternatively, use another group’s tube as a balance. It is very important that the centrifuge rotor is properly balanced!

Place caps on the conical tubes and insert the tubes on opposite sides of the rotor as shown in the figure. Secure the cover onto the rotor and set the centrifuge to run for 10 minutes at 5,000 rpm at 4OC. Press the ‘on’ button and stay by the centrifuge until it comes up to speed. If it makes any loud noises, stop it immediately and recheck the balance of your tubes. Once it is finished, remove your tubes and turn the centrifuge off – unless someone else will be using it.

You want to save the cells that are in the purple pellet at the bottom of the tubes. Decant the liquid into a waste receptacle by pouring it away from the pellet. The liquid (spent media) must be treated with a 10% bleach solution for 20 min before being discarded down the drain. The Erlenmeyer flask that your cells were growing in should also be treated with a 10% bleach solution for 20 min then washed as usual with Alconox and rinsed and then hung to dry.

While you are bleaching the waste – Weigh your pellet. Put the tube on the balance and then subtract the weight of an empty tube (or tare it). This is the **wet pellet weight** – record the value.

\_\_\_\_\_\_\_\_\_\_ g Wet pellet weight

For **Figure 3.** Take a picture of your cell pellet and post to your Protein Labs page

**Resuspend the cells in Phosphate buffered saline**

In a 15 ml conical tube, make a 1x PBS working solution from the stock PBS you made in the 1st Lab. Label your 1xPBS working solution properly. Add 2.5 ml of 1x PBS to the 50 ml conical tube containing your cell pellet and recap. Vortex the tube to obtain an even suspension of cells – there shouldn’t be any clumps. You can also pipette the solution up and down to obtain a homogenous suspension.

Remove a tube of lysozyme from the -20OC freezer and keep it in an ice bucket to de-thaw.

Then add stock lysozyme (50 ug/ul) to a final concentration of 1 mg/ml and vortex again.

Label the tube well and place in a rack in the -20OC freezer with the other VDS reagents and samples.

Draw a ‘tick mark’ on the lid of the lysozyme tube to denote that is has been thawed once and Replace lysozyme back in freezer.

**Clean your station. Dispose of any hazardous waste (e.g. bleached items)**

Lab Notebook – follow guidelines in the **LabNotebookvsLabReport.doc** that we have been using. Start with a brief objective. Each Methods step should be very detailed so as to allow another person to recreate your work. Remember that quick drawing or sketches add a lot of clarity to your notebook. End with a brief conclusion. Be sure to annotate the times you did the steps in your notebook along with the date.

Before you are done with the lab this week - you will need to post the images and captions from this lab online to your Protein Labs page on the Wikispaces site. A page with your name will be created where you can upload your pictures and a GOOD caption for each.

POST this information on your ‘PROTEIN LABS’ page on the the Wikispaces site

1. Plate Image
2. Flask Image
3. Pellet Image
   1. display your pellet weight in the caption of this figure.

Do you think that you beat the other groups in getting the most bacteria?

Lab Report – the lab report will be an online, combined lab report over 3 labs (protein expression, purification, & characterization). So, you do not have to turn in a lab report until the 3rd lab is done.

Lab Notebook: **Turn in your Lab Notebook when you have finished all of the steps and posted your images online.**